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## THE ACTION OF PHOMOPSIS CALIFORNICA IN PRODUCING A STEM-END DECAY OF CITRUS FRUITS<sup>1</sup>

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### INTRODUCTION

The aim of this investigation was to study the parasitism of *Phomopsis californica* Fawe., especially as to the manner in which the organism affects the different tissues of citrus fruits.

This fungus is similar to *Phomopsis citri*, which causes stem-end rot and melanose in Florida. *P. californica* was found by Fawcett (1922, 1924, 1926) to be the causal agent of decorticosis (shell bark) of lemon trunks and of a leathery, pliable stem-end rot of citrus fruits.

Of the various citrus fruits inoculated, lemons proved to be the most susceptible to this decay. The stem end, which is the usual place for beginning of decay under natural conditions, was also found to be the ideal place for infection under laboratory conditions. Wounds or punctures always facilitated infection.

It was noted that certain tissues of the lemon fruit were readily invaded by the fungus, while others remained free from invasion. The cells of the loose parenchyma tissues of the inner portion of the rind, known as the albedo, and those forming the axis of the fruit, known as the core, as well as the vascular bundles, were the elements most commonly attacked. On the other hand, the oil-bearing and

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juice-bearing tissues were almost free from the invasion. The effect of the fungus on the fruit was found to be partly mechanical and partly enzymatic.

A study of the enzymes excreted by the fungus during its attack was considered necessary to explain more completely the real nature of the decay.

## MICROSCOPIC STUDY OF DISEASED AND SOUND TISSUE

*Methods.*—Both free-hand sections and material imbedded in paraffin were used. On the whole, it was found that the best fixative was chrom-acetic-urea, composed of 1 per cent each of chromic acid, glacial acetic acid and urea in distilled water. Because of the wax that protects the epidermis of the lemon rind, penetration by the fixing fluid was generally slow. In order to prevent the inner parts of the material from breaking down, in warm weather, before the fixing agent reached them, the material was cut into small pieces and fixed at low temperatures. A period of two days was found necessary for thorough penetration; the renewal of the fixative once or twice during this time seemed to be helpful. To harden the material, especially the diseased lemon rind, it was left in 70 per cent alcohol for three days during dehydration. The material could then be preserved indefinitely in the same strength of alcohol for future use. Of several differentiating stains tested, Magdala red with light green proved to be the best. The mycelial threads became stained a deep pink, while the lemon rind tissues became green.

*Structure of the Normal Tissues.*—To understand the nature of the decay it was necessary to study in some detail the structure of the normal fruit. The lemon fruit may be considered as a highly modified berry. The mature rind is yellow throughout the external third or half of its thickness, the coloring being due to the presence of carotinoids and oil. The albedo or inner portion of the rind is white in color.

The ovary consists of a single whorl of carpels, the outer layers of which develop the rind and the inner the pulp. The outer layers of the carpels are united along the whole length of their edges to form the rind, while the inner layers fold towards the core of the fruit to form the segments, including their separating membranes (*see* Ross, 1890).

The mature epidermis, which is generally protected with a waxy substance, is composed of one layer of empty, colorless, thin-walled cells mostly rectangular in shape.

In citrus fruits the periderm lies immediately below the epidermis. Next below the periderm is a layer of thin-walled parenchyma cells. Both the parenchyma and the periderm contain many chromatophores. Next below these supporting layers is found the albedo with its loose parenchyma cells extending to the pulp. Between the two parenchyma layers are found more compact parenchyma cells, within which are the widely separated groups of bast fibers.

Any section through the lemon rind reveals three sizes of oil glands, mostly globular in shape. The larger ones are located immediately beneath the small depression of the rind, while the intermediate and small ones are between the large glands. We occasionally found a

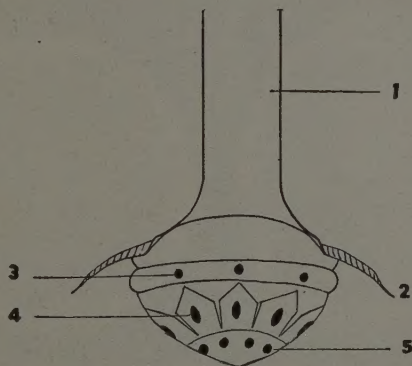


Fig. 1. Diagram of a lemon button; 1, stem; 2, cut calyx; 3, 4, and 5, projecting strands of the three circles representing the branching of the main group of vascular bundles of the stem.

fourth type of oil gland, which resembles a long-necked flask. Each type of gland originates from a mother cell, which divides rapidly. The thin walls of most of the middle cells are soon dissolved, thus enlarging the gland cavity. The protective wall of the oil gland is composed of closely compacted cells without intercellular spaces.

The wall of each segment is enclosed by a narrow, white sheath of parenchyma cells, which connects the albedo tissue with the core. Within the sheath are numerous vesicles, which contain the juice in large, thin-walled cells. The walls of the vesicles are thin and are composed of narrow, elongated cells arranged perpendicularly to the axis of the fruit. Each vesicle is attached to the concave side of its segment wall by a rather delicate stalk. There is much variation in the size and shape of the vesicles, which is due to position and pressure.



In studying the vascular bundles of the fruit it was found that as those of a twig diverge to form the main veins of the leaf, so do the vascular strands branch out to form the fibro-vascular strands of the system of coalesced carpels called the fruit. This divergence takes place in the receptacle, which is united with the persistent fleshy calyx to form the button of the fruit. Examination of the lower surface of a normal button where it is attached to the fruit reveals to the unaided eye three concentric circles of tooth-like projections (3, 4, and 5 in fig. 1). The button scar (the depression from which the button has been removed) shows the continuation of these three

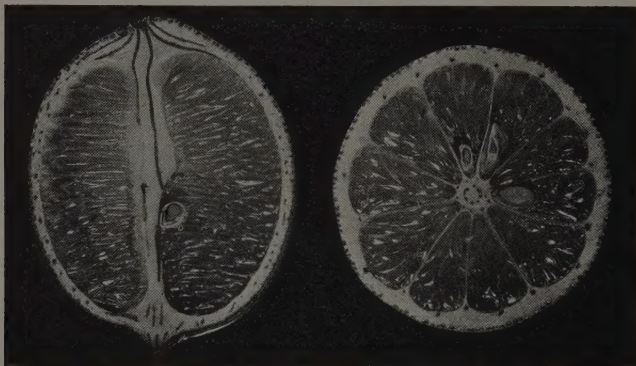


Fig. 2. A longitudinal and a cross-section of mature healthy lemons, with the vascular-bundle strands inked to show their distribution in the rind and core.

circles of bundles. As long as the button is attached to the fruit, the strands projecting from the button will be connected with the corresponding ones in the fruit. Between the button and the fruit tissues lies the abscission layer at which separation takes place when the button is pulled out. In the longitudinal section of the fruit (fig. 2), the strands of the main central circle are seen to continue downwards from the button into the core and the nearby tissues. The vascular bundles of the second circle of the button, however, diverge and proceed between the rind and the pulp, supplying mainly the latter. The strands of the third circle diverge still more to form a network through the rind. In the cross-section of the fruit (fig 2) most of the vascular strands appear to the naked eye as small specks scattered throughout the rind, between the rind and the segments, and in the core. There is always a main bundle found midway along

the side of each segment where it touches the albedo. This bundle is similar to the midrib of the unmodified lemon leaf. There is also a smaller bundle at the angle between each two segments (fig. 2).

The core is composed mostly of large spongy parenchyma cells, among which *Phomopsis californica* grows readily. In the outer portion of the core, extending from the stem to stylar end, are the strands of the main vascular bundle.

*The Diseased Tissue.*—The first external symptom induced by placing spores of *Phomopsis californica* in the button scar of mature lemons is the development of a characteristic pliable leathery circle just around the button. During the first week of development this circle enlarges gradually but slowly towards the stylar end without any visible discoloration. Later the invaded surface loses its natural color and becomes honey yellow, then ochraceous buff, and finally buff brown. Such changes vary somewhat with differences in temperature, humidity, and age of fruit. When the discoloration covers nearly one-fourth the area of the rind, a water-soaked belt can be seen advancing ahead of the line of discoloration. This belt is not sunken and its advancing margin is the border-line between the diseased and healthy tissues. This belt, the width of which varies from one-fourth to one-half inch, encircles the rind in a continuous but wavy form. It continues to advance towards the stylar end, followed immediately by pliability and discoloration, the entire surface of the fruit becoming buff brown in color. In some cases, under favorable conditions, a furrow-like depression opposite each segment of the fruit is an added symptom.

When spores of *Phomopsis californica* are sown in distilled water upon the rind of a sound lemon fruit, no infection ordinarily results.

In an experiment in which drops of water containing spores were placed on the uninjured surface of the rind most of the spores germinated, but the germ tubes failed to penetrate the rind. In experiments in which wounds were made through the outer oil-bearing tissue of the rind, however, infection took place and development of decay followed. Such decay took approximately three weeks to become visible on the surface of the rind. Once the hyphae reached, by means of wounds, the inner tissue of the rind, they progressed without difficulty. On the other hand, if the button scar was filled with the spore suspension, either in water or in nutrient solutions, no further wound was necessary to cause infection. A puncture or a drop of prune juice, however, hastened infection. The button scar evidently furnishes ideal conditions for the development and penetration of the fungus.

An examination of various tissues after the development of decay indicated that the fungus was not present in all parts of the affected portion. This was shown by transfers to glucose-potato-agar plates from the different zones of diseased lemons. For example, five transfers were made from a lemon (fig. 3) each consisting of nearly the same quantity of tissues taken approximately 2 mm below the epidermis. The five plates were incubated for a week under a bell jar at room temperature. Transfer 5, made from the sound area, and transfer 4, made from the water-soaked advancing zone, showed no

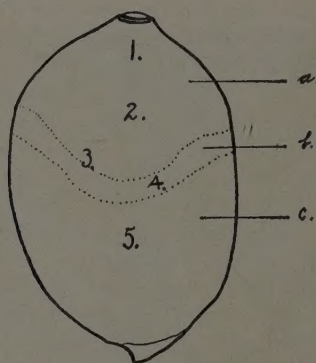


Fig. 3. Diagram of a lemon attacked by *Phomopsis californica*: a, discolored diseased rind; b, water-soaked advancing zone; c, healthy rind; 1 to 5, points of isolation (see text, page 158).

growth. Transfer 3, made from the advancing margin of discoloration, gave an excellent growth. Transfer 2, taken a little farther back, also gave a fair growth, while transfer 1, which was still farther back, gave no growth. This experiment was repeated three times with similar results. This result indicated that the advancing zone at 4 was free from the organism, that the margin of discoloration at 3 had the most active mycelium, that the tissue at 2, farther back, had less active mycelium, and that the tissue still farther back, at 1, had no live mycelium. As judged from microscopic examination of tissues at 1, showing the absence of mycelium, it is inferred that the mycelium had been dissolved.

When a diseased fruit was cut longitudinally, the internal pathological changes seemed to coincide, to a considerable extent, with the external symptoms (fig. 4).



The three zones, namely, the discolored, the water-soaked, and the healthy, could be recognized. The discoloration in the core advanced far ahead of the limit found in other tissues. In the rind the discoloration appeared to travel faster through the albedo than through the outer rind; the discoloration in the albedo was always in advance of the discoloration upon the surface. In longitudinal sections the more rapid advance in the albedo produced a tongue of discolored tissue projecting between the sound pulp and the sound outer rind.

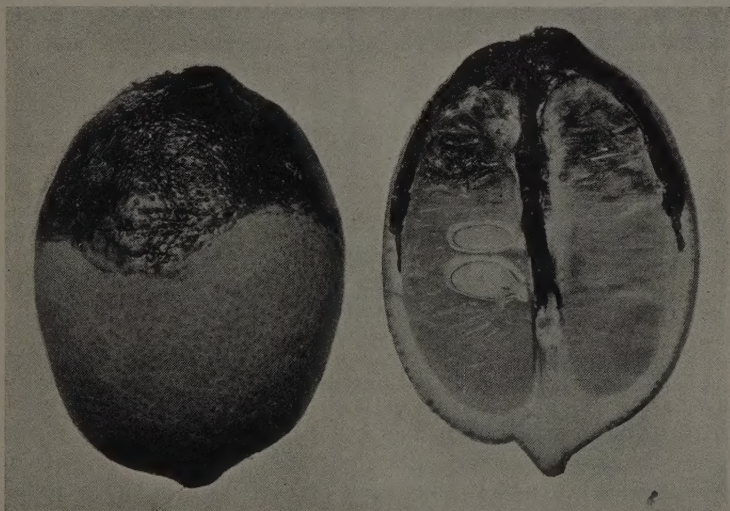


Fig. 4. External and internal symptoms of *Phomopsis californica* on a lemon fruit showing slightly affected pulp, advancing tongue of decay in albedo, and the decayed core.

This continued until the whole rind was entirely discolored. The pulp tissues seemed to be the least affected. Only in advanced cases did the walls of the segments and the walls of the vesicles next to them become discolored. The outer rind and the pulp tissues were usually firm. The parenchyma cells of the albedo, when severely affected, tended to become separated.

With the aid of a microscope, it became evident that the advancing water-soaked area was free from the mycelium. The pathological changes in a cross-section of the core just ahead of discoloration seemed to be mainly plasmolysis and maceration (fig. 5). Further examination of the slides revealed that the epidermis, the oil-bearing

tissues, and the vesicles containing the juice, were also free from the organism. No hyphae were found inside or in the neighborhood of epidermal cells, oil glands, or juice vesicles. The attack of the fungus seemed to be mainly confined to the spongy parenchyma cells of the core and the albedo. In such tissues bits of the mycelium were frequently seen inside the macerated cells, or along their cell walls. In later stages of decay, invasion of the vascular bundles in the core and the albedo was noted, but it was evident that the hyphae were most abundant in the parenchyma tissues surrounding these bundles. The attacked cells, besides being separated from each other along the middle lamellae, become plasmolyzed. Occasionally the walls of



Fig. 5. Cross-section through a core 2 mm beyond discoloration, showing mainly plasmolysis and maceration.

the penetrated cells were broken in one or two places. Where the mycelium came in contact with the walls, the latter decreased in thickness. When sections were made from rind which had been discolored for a considerable time, such as that close to the stem end of a half-decayed fruit, the mycelium was found to be dissolved. On the other hand, when sections were prepared from the newly attacked tissues, especially from those close to the water-soaked area, long mycelial threads could easily be detected.

The fungus exhibited a very strong deamidase reaction, as will be shown in the section on fungus enzymes. The large amount of ammonia formed in the process suggested that this substance might have some connection with the development of the water-soaked belt in the decaying fruit. Accordingly a preliminary test was made for the purpose of observing the effect of ammonia on sound fruit. A lemon was cut twice longitudinally at right angles through the core and once transversely, making eight equal portions. These were placed separately in bottles containing 4, 2, 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$ ,  $\frac{1}{16}$  and  $\frac{1}{32}$  per cent solutions of 28 per cent ammonia (sp. gr. 0.9) respec-



tively. All the flasks were corked tightly and were left at room temperature for observation. The first three solutions caused the rind to become dark brown in less than six hours, while the rest showed moderate effects. Solutions of  $\frac{1}{2}$  and  $\frac{1}{4}$  per cent developed on the rind the typical buff-brown color of stem-end decay, and caused maceration in both the albedo and the parenchyma cells of the core. The vesicles also were separated from each other. The last three solutions caused no change in color of the mature rind, but caused slow disintegration.

In another experiment solutions of 1 per cent, and one-half and one-fourth of 1 per cent of ammonia were drawn inside healthy mature fruits by means of a suction pump. A piece 3 mm in thickness was cut from the stylar end of a lemon and this end was fitted tightly in the neck of a suction flask. The button of the stem end was then removed, the exposed end wounded once with a scalpel, and the ammonia solution applied. A partial vacuum of ten pounds quickly drew the solution into the lemon. In this case even a 1 per cent solution developed internal and external symptoms similar to those caused by the disease. This would suggest the possibility that the water-soaked effect and the discoloration which follows the water soaking may be due in part to the by-products of enzymatic action, such as ammonia.

The discovery of the mycelium within and along the vascular bundles led to the following experiment. Six lemons, each with a stem one-half inch in length, were placed perpendicularly in such a position that the ends of their attached stems were immersed separately in a suspension of *Phomopsis* spores. In another six the attached stems were longitudinally split, and one-half of each was removed with its respective half of the button. These were treated in the same manner. This experiment covered a period of three weeks at room temperature, more of the suspension being added occasionally to the containers so that the cut ends were always in contact with the liquid. In those fruits which had normal stems the external symptoms of the disease developed gradually until the entire rind was discolored. In the fruits of the second set the symptoms appeared only on the sides which had connection with the suspension through the half stems. When samples of fruit were cut longitudinally, the internal pathological changes agreed with the external symptoms. Culture tests showed that only the discolored half contained the fungus, indicating that the mycelium advanced into the fruit along the vascular bundles on the half of the stem not cut away, from which it freely invaded the nearby tissues.

## RELATION OF THE KINDS OF TISSUE AND THEIR CONTENTS TO THE GROWTH OF THE FUNGUS

*Oil-bearing Tissues.*—It was shown above that the *Phomopsis* hyphae did not at first enter the oil-bearing tissues, but concentrated in the core and in the albedo. These facts led to the following experiments to determine whether the lemon oil itself might have anything to do with the absence of hyphae in the oil-bearing tissues.

The rind of a fresh healthy lemon was removed and was cut into longitudinal strips one-fourth inch in width. These strips were again cut so that the albedo layers were severed, as far as possible, from the external rind. The inner and outer strips were then placed separately in culture tubes, at the bottom of which moistened pieces of cotton had been previously inserted. All the tubes were then autoclaved and inoculated with the organism. In two weeks good growth of the fungus was observed in each tube, regardless of the portion of the rind use.

In a similar experiment, the same two parts of the rind were placed in culture tubes under as sterile conditions as possible and inoculated as before. Great care was taken to have the media sterile without applying heat. All the inoculated tubes of albedo developed an excellent growth of *Phomopsis* while the corresponding inoculated tubes of outer rind showed no growth. The tissues of the albedo first became brown in color, then were nearly covered with mycelium, and finally were dotted with pycnidia. The inoculated tubes containing the outer rind showed no growth of any kind, remaining the same as those not inoculated.

In a third experiment the fungus failed to grow when 1 per cent of pure lemon oil was added to a carbon-free medium; this indicates that the fungus was unable to utilize the oil as a sole source of carbon.

The last result led to a fourth experiment in which 1 per cent of lemon oil was added to a full nutrient solution in flasks and inoculated. The flasks were frequently shaken so that the oil was kept mixed with the other nutrients for a considerable time. No growth took place in these cultures, but very good growth occurred in check flasks of the same medium which had received no oil. In another trial strips of albedo and oil-bearing tissue were prepared as in the second experiment and pure lemon oil was added to the strips before inoculation. No growth took place in any of the tubes. The albedo, which

was normally a good medium for the fungus, failed to support growth on the addition of the lemon oil.

It therefore appeared probable that the oil-bearing tissues of a normal rind are protected by the oil from invasion by the fungus, which could thrive well on tissues free from the oil, such as the core and the albedo. In the first experiment, the fungus also grew well on the oil-bearing portion of the rind after being autoclaved, probably because the oil had been volatilized by heat.

*The Juice-bearing Tissue.*—During the present investigation a considerable number of lemon fruits attacked by *Phomopsis californica* were examined, in both early and advanced stages. To the naked eye the pulp of these fruits seemed to escape the attack, especially in the early stages. When examined microscopically, however, the walls of the segments were found to be infected, while the walls of the vesicles within were usually free from invasion. Only in the very advanced stages were the vesicular walls attacked. Even in such advanced cases the mycelia were found in the walls of the vesicles, but never in the juice itself. This last observation regarding the juice was similar to that regarding the oil and suggested further experiments.

Pulp of a sound lemon from which the core had been removed was squeezed in cheesecloth and washed several times with water until it was freed of nearly all its natural juice. This pulp supported a very good growth when it was inoculated with the *Phomopsis* organism, while untreated pulp with all the juice left in it permitted only a very poor growth.

In another experiment, no growth was obtained when lemon juice was used as a medium, while both orange juice and prune juice gave an excellent growth. It was found later that the spores of *Phomopsis californica* were unable to germinate in undiluted lemon juice; but when the latter was diluted 1 to 1 a few spores gave rise to germ tubes.

When citric acid was added to a full nutrient solution at the rate of 4 per cent, it inhibited the growth of the fungus.

In another experiment, six flasks with 50 cc of carbon-free nutrient solution containing 1 per cent tannic, malic, oxalic, citric, formic, and tartaric acids, respectively, were inoculated with a *Phomopsis* pycnidium.

In the first few weeks a slight growth appeared in the flask containing the tannic acid. Later the solution turned brown and then black, and finally a good growth developed with pycnidia formed over a solid mat of mycelium. In the malic acid medium a fair growth was obtained, but the pycnidia were lacking. In the oxalic acid a



poor growth developed. In the citric, formic, and tartaric acids, however, no growth took place.

Camp (1923) found that none of the fungi (including *Phomopsis citri*) which commonly attack citrus fruits, could thrive on citrate as the sole source of carbon.

Since even the addition of citric acid to full nutrient solutions checked the growth of the fungus, it seems highly probable that the acid is responsible for the protection of the juice-bearing tissues from invasion.

## THE ENZYMES PRODUCED BY THE FUNGUS AND THEIR RELATION TO THE DECAY

The important rôle played by enzymes in plant-disease phenomena has long been recognized and studied, and the subject is ably summarized in the recent papers of Hawkins and Harvey (1919) and Harter and Weimer (1921). In the course of the investigations on the *Phomopsis* rot it became evident that a survey of the enzymes of the fungus was necessary to help elucidate the nature of the parasitism. Accordingly, after a preliminary test for the presence of enzymes in an extract made from the fungus, the enzymes thought to be most directly concerned in the intimate parasite-host relationship were tested for.

*Culture Media*.—As a liquid medium, Duggar's (-1909) standard nutrient solution was prepared with the addition of a small quantity of sodium chlorid as follows:

Ammonium nitrate .....	1.0 gram
Dihydrogen potassium phosphate.....	0.5 gram
Iron chlorid .....	trace
Sodium chlorid .....	trace
Magnesium sulfate .....	0.25 gram
Cane sugar .....	5.0 grams
Water .....	100.0 cc

Cane sugar was omitted when a non-carbon medium was needed and 1.5 per cent of "Bacto" agar was added when a solid medium was required.

A solid non-carbon medium, which did not support the growth of microorganisms, was prepared in large quantities and is referred to as "stock medium" in the following discussion.

*The Preparation of the Mycelial Extract and Mycelial Powder*.—Pure cultures of *Phomopsis californica* were grown in Czapek's nutri-

ent solution in large flasks. In two months thick mats were developed. These were washed gently with distilled water, and ground in a porcelain mortar with approximately an equal weight of clean quartz sand. The resulting fine pulp with an equal volume of sterile water was transferred to a 500 cc Erlenmeyer flask, a few drops of toluene

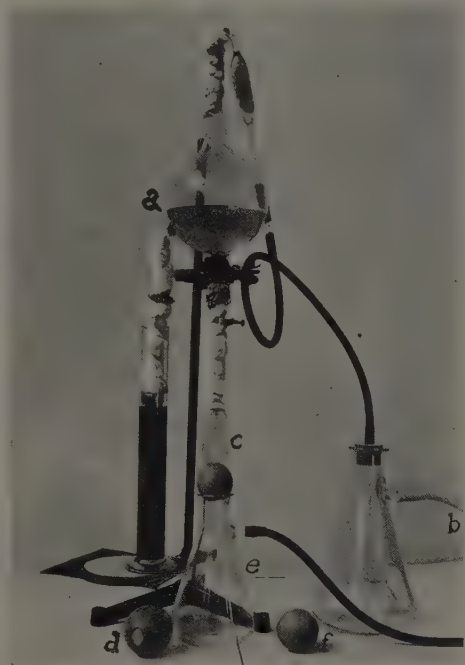


Fig. 6. Filter apparatus used in testing the effects of the mycelial extracts on the healthy tissues of the lemon. In this apparatus the liquid is drawn up through the Mandle filter candle into the large flask (a) under five pounds vacuum at b, which is connected to a water-vacuum pump. The sterilized filtrate runs off below by means of a stop-cock and the outlet glass tube, which is enveloped in the larger tube, c. The only possibility of contamination is from the air. The connecting tubes are wrapped with thick layers of cotton as a precaution against such contamination. Before using, the apparatus is sterilized in an autoclave; d, lemon ready to be treated; e, filter flask connected with a water-vacuum pump; f, lemon with exposed vascular bundles of the fruit after the removal of three millimeters from the styler end to facilitate suction.

added, and the whole incubated at 30 degrees C for several hours. From time to time the flask was vigorously shaken, and finally the material was allowed to settle. The supernatant liquid was siphoned

off and filtered through a filter paper, and then sterilized by drawing it through a Mandler filter candle under five pounds partial vacuum (fig. 6). The apparatus used was an imitation of the one described by R. E. Smith (1917). The apparatus with the sterile mycelial extract was stored in the culture room away from the bright light.

Mycelial powder was also prepared from thick mats of the fungus by the Acetone-Dauer-Hefe method of Albert, Buchner, and Rapp (1902) with a few modifications. The mats were ground in a mortar to a fine pulp, four times their volume of acetone was added, and the whole stirred five minutes. The material was drained as dry as possible on a suction filter and the process was repeated, using a quantity of acetone equal to that of the mycelial pulp. The mycelium was finally similarly treated with an equal volume of ethyl ether and spread out to dry for 12 hours in an oven at 30° C. The desiccated material was then ground to a fine powder and stored in a brown glass bottle.

*Effect of the Mycelial Extract on Sound Lemons.*—Early during this study strips from the rind were placed in the extract for observation, but later the entire fruit was used, the extract being placed in a puncture 1 inch deep made through the stem end. By the method described in connection with the experiments on the effect of ammonia, definite quantities of the extract were drawn into fruit. When the active extract was used, the sterilizing apparatus was employed in such a way that one drop at a time filled the button scar of the fruit (fig. 6). As checks, some extract inactivated by boiling, and some distilled water were drawn into other fruits.

TABLE 1

EFFECT OF PULLING MYCELIAL EXTRACT INTO FRUIT UNDER PRESSURE

Quantities	Active extract	Boiled extract	Distilled water
2 drops	Very slight effect	No effect	No effect
5 drops	Much effect	No effect	No effect
10 drops	Typical disease symptoms	No effect	No effect

Many ripe lemons were first treated externally by dipping in 5 per cent solution of sodium borate for one minute. After a few days 27 fruits were selected from those which seemed normal and 3 fruits each were treated by the methods shown in table 1. After such treatment all the fruits were incubated at 30° C for three days.



After the incubation period each fruit was examined carefully, and then was cut longitudinally for study of the effect on the internal tissues.

The first three fruits, treated with 2 drops of active extract, showed only very slight effect. The core tissues were affected more than the rest and no external changes were visible. The second three fruits, with 5 drops of active extract, developed to some extent internal and external changes which resembled those of an early attack of the disease. The next three fruits, which received 10 drops each, showed characteristic symptoms both internal and external. The epidermis, however, developed a water-soaked appearance instead of the usual buff brown. This condition was almost typical of that of the advancing zone that appears ahead of the discoloration in tissues attacked by the fungus, as previously described. Although these symptoms appeared in a shorter time than with fungus inoculations, the general disintegration among the internal tissues, and the leathery pliable condition of the surface was practically the same as in fruits inoculated with the fungus. When the vascular bundle strands were traced down through the tissues, it was noticed that they were darker in color than the adjacent tissues. The tissues adjacent to the bundles were more affected than any of the other portions. The next nine fruits, which received the boiled extract, did not show any of the changes described, regardless of the quantities drawn into them. The fruits which received the distilled water appeared normal in every respect.

It was evident, therefore, that the mycelial extract was capable of developing some of the characteristic symptoms of the disease, and that the amount used had a direct relation to the degree of the development of the symptoms.

*Testing for Cytase.*—The breaking-down of cellulose by micro-organisms has been studied by Kellerman and McBeth (1912), McBeth and Scales (1913), Cooley (1914), and many others.

With *Phomopsis californica* the following experiments were carried out: Swedish filter paper was soaked in the carbon-free nutrient solution and inoculated with pycnidia in six petri dishes. Good growth took place, showing that the fungus was able to utilize the cellulose of the filter paper as the only source of carbon.

To obtain more accurate results, pure cellulose precipitate was prepared as described by Cooley (1914) and added to the stock medium. The resulting solid cellulose medium was then autoclaved, plated, and inoculated with pycnidia. The good growth that resulted was further evidence of the ability of the fungus to attack cellulose.

In another experiment 5 cc of the mycelial extract was added to tubes of cellulose medium along with a few drops of toluene for disinfection. As a check, boiled extract was used in tubes in the same way. The cellulose agar columns in those tubes receiving the active extract were cleared to an average depth of 4 mm, while those of the check tubes receiving boiled extract remained uniformly cloudy.

The experiments above, as well as the microscopical observations previously described, showed the ability of the fungus to attack celluloses, and indicated that the enzyme cytase was present in the mycelial extract.

*Testing for Pectase and Pectinase.*—The enzyme which is capable of hydrolyzing pectic compounds of the cell wall is called pectinase (pectosinase by some investigators), while the one causing their coagulation is termed pectase. Since it was found in microscopical examinations that the middle lamellae, especially those of the albedo cells, were dissolved, the demonstration of pectase and pectinase was attempted experimentally.

A commercial preparation of pectin (Certo) as a substrate gave negative results for pectase when tested with both the mycelial extract and the powder; it was not coagulated by either. In demonstrating the actions of pectinase many investigators have worked principally with mycelial extracts. In this investigation not only the mycelial extract but also the liquids in which the fungus had grown were tested for this enzyme.

Further experiments were conducted to determine the macerating power, or rather the pectinase action, on four different tissues. Both the mycelial extract and the powdered mycelium were used. Discs of lemon rind, potato, carrot, and beet were cut into uniform thickness and shape (17 mm in diameter and 5 mm thick) by means of a cork borer and a knife. In each of twenty 150 cc Erlenmeyer flasks were placed two discs of one of the four substrata, a few drops of toluene and 25 cc of the mycelial extract or water. Five flasks were used for each substratum, two containing the active extract, two the boiled extract, and a fifth distilled water. The flasks were kept at 30° C.

Only the discs in the active, unheated extract showed evidence of maceration. The extent of maceration was judged by the relative ease of rupture of the discs by means of dissecting needles, and by the numbers of separated cells revealed by the microscope. The beet discs were completely disintegrated in 48 hours, and the anthocyan disappeared in 24 hours. This indicated that the active extract not only destroyed the middle lamella, but also made the protoplasts more

permeable and destroyed the pigment that exosmosed. While the boiled extract caused no maceration or destruction of the pigment, it did permit diffusion of the pigment into the surrounding water. The carrot discs in the active extract showed only slight maceration in 48 hours, and this only on the perimetrical part. The enzyme attacked the potato discs very vigorously and the cells showed complete separation in 24 hours. Lemon-rind discs resisted dissolution, exhibiting but a slight effect at the perimeter. This work was repeated, using the culture medium in which fungus had grown. The results were similar to those with the extract; that is, maceration was effected only by the unheated material. The production of pectinase by *Phomopsis californica* was therefore demonstrated.

*Testing for Invertase and Maltase.*—Since disaccharides as well as cellulose are important constituents of lemon rind, as pointed out by Bartholomew and Robbins (1926), it was thought advisable to test for invertase and maltase also.

One gram of pure sucrose was added to 100 cc of the stock medium as the sole source of carbon. To another 100 cc of the stock medium one gram of pure glucose was added as the sole source of carbon. Petri dishes with both media were inoculated with a few spores of the fungus. In one week growth in the glucose cultures was much ahead of that in the sucrose plates. Gradually, however, the development of the fungus in the sucrose plates became more vigorous, and the final results were similar to those with the glucose plates. This sort of development gave an indication that it was necessary to invert the sucrose into glucose and fructose before it became available.

In a second experiment 5 cc of the mycelial extract was added to 50 cc of a 1-per-cent sucrose solution with a few drops of toluene as an antiseptic. As a control, boiled mycelial extract was used in the same manner. A duplicate set was made. After 24 hours at 30° C the reducing power of each solution was determined. Use was made of the rapid volumetric method of titrating the unknown directly against Fehling's solution, 10 cc of which was considered to be completely reduced by 0.05 gram of glucose (Haas and Hill, 1917). Table 2 shows the results obtained.

These experiments showed that *Phomopsis californica* was able to produce the enzyme invertase.

The presence of maltase was demonstrated in a way similar to that of invertase. The growth of the fungus was rather slow at first, but eventually became vigorous, indicating that the organism hydrolyzes the disaccharide to dextrose before assimilating it.



In a second experiment 25 cc of a 1 per cent maltose solution was treated with 2 cc of mycelial extract and a few drops of toluene added as an antiseptic. As a control both the boiled extract and distilled water were added to another set of flasks, each of which contained

TABLE 2  
QUANTITATIVE STUDY OF THE INVERTASE IN THE MYCELIAL EXTRACT

No.	Myc. extract added to 50 cc of 1 per cent sucrose solution	Substratum used to reduce 10 cc of Fehling's	Glucose in substratum	Glucose due to invertase
	cc	cc	per cent	net per cent
1	5	5.6	0.9	0.70
2	5 (boiled)	25.0	0.2	
3	5	5.8	0.86	0.66
4	5 (boiled)	25.0	0.2	

the same amounts of sugar solution and antiseptic. After 48 hours at 30° C. the reducing power of each solution was determined by direct titration against 10 cc of Fehling's solution as shown in table 3.

The above experiments showed that *Phomopsis californica* had the ability to produce maltase.

*Testing for Emulsin.*—Emulsin was discovered in *Aspergillus niger* by Bourquelot (1893) who also discovered it in 25 species of higher fungi. The presence of emulsin in *Phomopsis* mycelia was determined in a preliminary way by the ability of the fungus to use the glucoside arbutin. Owing to the solubility of glucosides, the agar containing them is clear and the action of the enzymes could be easily detected. Arbutin, if hydrolyzed, yields glucose and hydroquinone, and the latter stains the clear medium with a brown color.

TABLE 3  
QUANTITATIVE STUDY OF THE MALTASE IN THE MYCELIAL EXTRACT

No.	Amt. of myc. extract added to 25 cc of 1% maltose solution	Amt. of substratum used to reduce 10 cc of Fehling's	Increase in reducing power due to maltase
	cc	cc	per cent
1	2	5.0	62
2	2 (boiled)	8.1	
3	2 distilled, H <sub>2</sub> O	8.2	

Arbutin added to the stock medium at the rate of 1 per cent supported an abundant growth of the organism. A brown stain soon appeared in this medium, indicating hydrolysis of the glucoside and the presence of the enzyme emulsin.

In a second experiment 5 cc each of the liquid in which the fungus had grown was added to a set of 5 tubes of arbutin medium. As a check, 5 cc of the boiled solution was added to another set. In the first set only were the agar columns stained brown, indicating that the arbutin had been hydrolyzed to glucose and hydroquinone.

A third experiment was conducted in which the mycelial powder previously described was employed in a thin layer over the surface of each arbutin-agar column. As a check some of the powder was inactivated by heat before use. The brown color, due to hydroquinone, was found to diffuse down to the bottom of the columns in the tubes treated with unheated mycelial extract, while the checks remained unchanged.

A fourth experiment, in which the activity of emulsion was estimated by the determination of the reducing power of the hydrolytic products, was conducted as follows: In each of 4 Erlenmeyer flasks was placed 25 cc of 1-per-cent arbutin solution. Also to each flask 0.25 gram of the mycelial powder and a few drops of toluene were added. The powder of the third and fourth flasks was inactivated by heat before use. The four flasks were shaken vigorously for one minute. In three days at 30° C the solutions in the first two flasks turned brown, while those of the other two remained clear.

It required only 5.4 and 5.6 cc respectively of the solution from the first two flasks, to reduce 10 cc of Fehling's solution, while more than 25 cc of the substrate of the last two flasks were required to cause the same action. This experiment indicated that *Phomopsis californica* had both intracellular and extracellular emulsin.

*Testing for Inulase.*—A 5 cc portion of mycelial extract and a small amount of mycelial powder was added respectively to 25 cc quantities of 1-per-cent inulin solution in Erlenmeyer flasks. After an incubation period of 3 days at 30° C the materials were tested for reducing sugars. All failed to reduce Fehling's solution, proving that *Phomopsis californica* does not produce the enzyme inulase under the conditions used.

*Testing for Proteases.*—As protoplasm consists largely of proteins these occur in all plants. Filamentous fungi, especially *Aspergillus* and *Penicillium*, have been commonly used to demonstrate proteoelastic reactions. Bourquelot and Herissey (1895) were the first to discover the protein-digesting enzymes in *Polyporus sulphureus*.

With *Phomopsis californica* experiments were conducted, using fibrin, gelatin, casein, and albumen as substrata. Powdered blood fibrin was added to the stock medium in the ratio of 1 per cent. Care was taken to shake the medium while pouring so that the protein

particles would be as evenly distributed as possible. Good growth took place on petri dishes of this medium, indicating that the fungus was able to utilize the fibrin as the only source of carbon, breaking it down with the aid of protease.

In another experiment the mycelial powder and red fibrin were used according to the method described by Reed (1913), in which the fibrin grains were stained in 1-per-cent Congo red and the color fixed by immersion in boiling water. The proteolysis was measured then by the amount of stain liberated into the solution.

The mycelial powder in neutral and alkaline solutions containing 20 cc of water and 2 grams of red fibrin showed a greater amount of red color than in an acid solution. The powder after being heated gave no color under the same conditions.

In a third experiment Rumbold's (1908) gelatin in plates was inoculated with fresh mycelia. After six days a good growth was noticed, with a liquefied circle 1 inch in diameter indicating a protease reaction.

In a fourth experiment the presence of erepsin was demonstrated by its hydrolytic action upon 1-per-cent casein in the stock medium. Good growth resulted in a week after inoculation with mycelium, and the secretion of erepsin was so uniform and abundant that a wide band of dissolved casein enclosed the margin of advancing hyphae on petri dishes.

Egg-albumin-agar prepared as recommended by Reed (1913) was used to test for the production of trypsin. Good growth, similar to that on the casein medium, took place, and a halo of clear agar formed ahead of the advancing mycelia; this shows that *Phomopsis californica* excretes trypsin.

*Testing for Amidases.*—Amidase is the enzyme which hydrolyzes acid amides to ammonia and organic acids. Accordingly, qualitative and quantitative tests for ammonia in the substrate, after incubation with the fungus or its enzyme preparations, were employed to demonstrate the presence of amidase. Since the ammonia formed produces an alkaline reaction which completely dominates the small concentration of H-ions contributed by the weakly ionized organic acids, any indicator which gives a brilliant color in the alkaline range may be used in the qualitative test. Rosolic acid, as recommended by Crabill and Reed (1915) was used; this gives a brilliant red color above pH 8.

In stock solution with 1 gram of asparagin and 1 cc of 2-per-cent rosolic acid in 100 cc of solution, kept at 20 to 25 degrees C, the



fungus grew slowly during the first few days, but during the second week abundant mycelia were developed, covering a circular area 1 inch in diameter. The rosolic acid, which gave the medium a yellow tint, turned red during the first day when the asparagin was attacked by the *Phomopsis* mycelia. During the second day the red color diffused readily all around and ahead of the advancing hyphae. During the third day there was no trace left of the yellow color in any of the transfers. In the controls, however, where no asparagin was added to the stock medium, no growth took place, and the yellow color of the rosolic acid was unchanged.

The above experiment was repeated, using in this case the mycelial powder, which was placed at the center of each petri dish. In an hour the unheated powder turned the yellow medium red, while in a similar test heated powder showed no effect.

In a third experiment mycelial extract was placed in shallow wells 2 mm in diameter and 3 mm deep. At the end of one-half hour at 30° C the cherry-red color diffused all around the surfaces of each well, and the red color invaded all the area of each plate in a day or two. Similar wells in the medium filled with the extract after it had been boiled gave no red color.

In a fourth experiment twelve test tubes of rosolic-acid medium were treated in three different ways. The first four tubes were each inoculated with a pycnidium. The pycnidia of the third and fourth tubes were heated over the flame before use. To each of the fifth, sixth, seventh, and eighth tubes 0.1 gram of the mycelial powder was distributed over the exposed surface. The powder of the seventh and eighth tubes was heated. To each of the remaining four tubes 5 cc of the mycelial extract was added; the extract of the last two tubes was boiled before use. After one week at 25° C the six controls, to which the heated materials were added, gave negative results, but the other six tubes gave positive results in different proportions. The red color diffused to the bottom of the two test tubes having the mycelial extract, while it diffused to a depth of only 2½ cm in the case of the pycnidia, and to twice as much in case of the mycelial powder.

A fifth experiment to measure the amount of ammonia liberated by the enzyme amidase was conducted as follows: to 25 cc of a 1-per-cent solution of asparagin in a 250-cc Erlenmeyer flask were added 25 grams of mycelial powder and 1 cc of toluene. As a control, the fungus powder used in this experiment was first autoclaved. Both the controls and the flasks with the active enzyme were run in dupli-

cate. After 24 hours at 30° C, the contents of each flask was filtered through a filter paper. Ten cc of each of the four filtrates was transferred to four test-tubes, and the ammonia was determined by the aeration method of Folin, with the tubes connected in series, so that four aerations could be made at the same time. Folin tubes with perforated bulbs were used for aerating the media and for delivery of the  $\text{NH}_3$  into each receiving tube containing 50 cc of 4-per-cent boric acid. To prevent excessive foaming two drops of kerosene were added to each of the four tubes containing the 10 cc of medium. The medium was made strongly alkaline to phenolphthalein by the addition of one drop of a saturated NaOH solution just before connecting each tube with its receiving tube containing the boric acid. A filter pump was connected to the last receiving tube and the solutions were aerated for 24 hours at the rate of approximately 50 liters of air an hour. The receiving tubes were titrated directly against standard sulphuric acid, brom-phenol-blue being used as an indicator. Table 4 shows the results obtained.

These experiments showed that *Phomopsis californica* possessed intracellular and extracellular amidase.

TABLE 4

AMMONIA PRODUCED BY THE ACTION OF AMIDASE OF PHOMOPSIS CALIFORNICA UPON ASPARAGIN

Flask		NH <sub>3</sub> liberated
1	25 cc of 1-per cent asparagin and 0.25 gram of mycelial powder.....	2.26 mg
2	Same as flask 1.....	2.10 mg
3	Same as above but the powder inactivated.....	0.93 mg
4	Same as flask 3.....	0.97 mg

*Testing for Lipase.*—Oil being a component of the lemon rind, it was thought advisable to test for lipoclastic ability in the fungus. First, use was made of litmus-cream-agar prepared, as recommended by Crabill and Reed (1915), by adding 10 cc of cream to 50 cc of distilled water and 40 cc of 10-per-cent gelatin solution. Blue litmus solution was then added to give the medium a deep blue color. The material was steamed one-half hour and then cooled and inoculated with mycelium of *Phomopsis*. After an incubation of one week the litmus medium remained blue, indicating at least that an insufficient quantity of fatty acids had been formed to raise the H-ion concentration of the medium to pH 5.5, the point at which the indicator turns pink.

This qualitative test was followed by quantitative tests in which 1-per-cent lemon-oil emulsion, methyl acetate, and ethyl acetate were used as substrates.

Fifteen cubic centimeters of each medium was used and 0.1 gram of the mycelial powder was added. Toluene was used as antiseptic. For the checks the power was inactivated by heat before use. After the incubation period the material of each flask was filtered and the acidity was titrated with N/10 NaOH, using phenolphthalein as an indicator. The average results of the duplicate experiment are given in table 5.

TABLE 5  
LYPOLYTIC ACTION OF THE MYCELIAL POWDER

Flask	Substrate	Mycelial powder	NaOH to neutralize 10 cc substrate
			cc
1	Methyl acetate.....	Active.....	5.3
2	Methyl acetate.....	Inactivated.....	0.2
3	Ethyl acetate.....	Active.....	5.75
4	Ethyl acetate.....	Inactivated.....	0.2
5	Lemon-oil emulsion.....	Active.....	0.45
6	Lemon-oil emulsion.....	Inactivated.....	0.1

The above experiments showed that *Phomopsis californica* did not exhibit any extracellular lipase, yet it seemed to contain an intracellular one. This was demonstrated chiefly by the inability of the fungus to grow on or use oils, while its mycelial powder showed a lypolytic action, as seen in table 5.

## DISCUSSION AND CONCLUSIONS

As described in the foregoing pages, the water-soaked belt ahead of the discoloration on the diseased fruit was always found to be free from mycelium of the causal organism. It is probable that some soluble substance produced by the fungus diffuses through the tissues and kills the host cells some distance ahead of the mycelium. The pathological changes in the advancing belt are mainly plasmolysis and maceration. Both the mycelial extract and the liquid in which the fungus had grown developed considerable enzymatic activity. Healthy tissues when acted upon by these liquids showed typical characteristic symptoms of the disease. These liquids when boiled, however, lost their ability to produce any of the symptoms of disease.

The fact that the mycelium, which is abundant at the margin of discoloration in the affected rind, later breaks down and dissolves when it is left far behind the advancing margin, and disappears entirely in old affected tissues, led to the conclusion that the enzymes and their by-products are probably able to dissolve these older hyphae.

In regard to the manner of invasion, it was shown that the infection always takes place through the stem end or wounds. Once the fungus gains entrance to the inner tissues of the fruit it develops steadily. The conclusion to be drawn from this is that the uninjured rind acts as a barrier. Since it was found that in diseased lemons the fungus was absent in the outer layers of the rind and was usually abundant in the albedo tissues, it became evident that the outer layers of the rind were in some way unsuited to growth of the fungus within them. As the oil glands are found only in the outer layers, and since the oil inhibits the fungus when added to susceptible tissue and cannot be used by the fungus even in small quantities as the only source of carbon in cultures, it seems probable that the presence of this oil is the main barrier to invasion.

It was pointed out that the fungus enters the fruit mainly through the exposed vascular strands and that the hyphae usually concentrate in the nearby parenchyma. These strands are abundant in the albedo and the core, both of which are free from the oil glands.

In the pulp the main protection against invasion by the mycelium may be attributed to the high acidity of the juice. The percentage of citric acid may be as high as 8.4 per cent (Haas, 1917) and the H-ion concentration of lemon juice extracted from thoroughly macerated pulp may range from pH 2.2 to 4.4 with an average of 2.31 for a large number of determinations from mature lemons (Bartholomew, 1923). Occasional tests of many of the media on which the fungus mats had grown showed an H-ion concentration of pH 4.7 to 5.2. Further evidence was obtained by testing the organic acids as the sole source of carbon, when it was found that growth developed only in the flasks having pH values of 7.6, 3.6, and 3.2 respectively. No growth took place at pH 2.6 and lower values. Since the lemon juice has an average pH value of 2.31 it seems probable that this high acidity protects it from the growth of the fungus, and probably also to a certain extent protects the juice-bearing tissues from invasion.

In a study of the enzymes produced by *Phomopsis californica*, cytase, pectinase, invertase, maltase, emulsin, proteases, amidase and lipase were found to be present. Pectase and inulase were tested for, but not found. Both the mycelial extract and the powdered mycelia



failed to coagulate pectin. In case of inulase, the fungus could not use the inulin as the sole source of carbon; neither the mycelial extract nor the powdered mycelia could hydrolyze inulin, as was shown by the inability of the treated media to reduce Fehling's solution. This shows that the fungus lacks both intracellular and extracellular pectase and inulase.

The growth of a fungus on a solid medium containing cellulose as the only source of carbon was indirect proof that this fungus secretes cytase which hydrolyzed the cellulose.

The ability of *Phomopsis californica* to attack cellulose was evident from its growth on pure filter papers. Microscopically, however, it was seen that the cytase in *Phomopsis californica* was limited since the cell walls were only slightly attacked. The effect upon the cell walls was much less than that produced by certain other fungi such as *Botrytis*.

With the aid of the microscope, the maceration due to the action of pectinase on the middle lamellae was seen very clearly. Even previous to the invasion by the hyphae the cells were widely separated from each other in the water-soaked advancing zone. This is shown clearly in figure 5, which was made from a core in the water-soaked area a few millimeters below the pioneering hyphae.

Since the cell walls were found to be acted upon only after they come in contact with the hyphae, and since their thickness was found to be the same in the water-soaked area and the healthy tissues, it may be concluded that the pectinase enzyme is secreted prior to and more abundantly than the cytase. This fact also may explain to some extent the pliability of the attacked rind. If cytase were very abundant the walls would be dissolved and the tissues would become soft instead of pliable.

In testing for enzymes which attack sugars it was found not only that the mycelium was able to utilize either sucrose or maltose as the only sources of carbon, but that the fungus powder vigorously hydrolyzed these sugars. Therefore, invertase and maltase were evidently present.

In testing for glucoside-splitting enzymes it was shown that emulsin was abundantly secreted. It is known that the glucoside hesperidin becomes evident in frozen citrus fruits. The widely-distributed astringent compounds known as tannins are also glucosides and may be found in very small quantities in citrus fruits. It has been shown that of various organic acids tested as the sole source of carbon, tannic acid gave the best growth. Tannins could be readily hydrolyzed, yielding some glucose for the nutrition of the fungus.

Early in this investigation it was found that the living fungus mycelium was unable to use lemon oil as a sole source of carbon, but when the mycelium was powdered to test the intracellular enzymes, it was found to have the ability to break down esters, as well as the lemon-oil emulsions, into acids (table 5). This indicates that, although the fungus appears to lack lipase as an extracellular enzyme, it contains this enzyme to some extent intracellularly. Since this enzyme remains within the living fungus cells it would not be expected to act upon the oil of the oil-bearing tissues, and this oil would therefore remain as a protection against invasion by the young advancing hyphae. It is possible that in the older diseased tissues, where the fungus cells break down the intracellular lipase may be liberated and thus be able to act upon the oil.

The general conclusion regarding the enzymatic activities of this fungus based on the foregoing experiments, may be summed up as follows:

Once the fungus gets into contact with either the albedo or the core tissues, the pectinase which it secretes enables it to dissolve the middle lamellae and cause maceration. This may be followed by secretion of cytase, enabling the fungus to get in contact with the protoplasm. Inside the cells the sugars may be used as a source of energy through the agency of carbohydrases, such as invertase and maltase. In the meantime proteases may be secreted, thus breaking down the proteins into amino acids and amides. The latter may then be acted upon by the enzyme amidase, which causes the liberation of ammonia. As the decay proceeds the young hyphae continue to grow and produce more and more enzymes. These enzymes and their by-products accumulate and diffuse ahead of the pioneering hyphae, thus paving the way for a more rapid invasion.

## SUMMARY

In this paper, in which the effect of *Phomopsis californica* on the tissues of the lemon fruit was studied, there are three topics considered, namely: A comparison between the tissues of normal and diseased lemons, the relation of the oil-bearing and the juice-bearing tissues to the growth of the fungus, and the enzymatic activities causing the decay.

A method of preparing and staining the diseased tissues is given which proved to be very satisfactory. Magdala red and light green were used as differentiating stains.

In studying the anatomy of the fruit, the vascular bundles, which are the principal channels for the invasion of the fungus, were found to branch into three circles at the button (receptacle), just before entering the fruit. The first circle proceeds straight into the core. The second follows between the rind and pulp, while the third diverges towards the rind and forms a net of strands.

The oil glands, which are found only in the outer layers of the rind, appear to protect that portion against invasion. In a similar way the protection of the pulp appears to be due to the high acidity of the juice it contains. The albedo and the core, which are free from oil glands and contain the greater part of the vascular bundles, were found to be the most affected portions of the fruit.

The nature of the decay was studied by means of tests for the presence of various enzymes in the mycelium and its products. To study the effect of such enzymes on the lemon tissues, a method was developed by which certain quantities of sterile mycelial extracts could be drawn into the fruits. Only the enzymes likely to be most important were tested for. The methods commonly used in identifying such enzymes were employed.

Concerning the carbohydrases, positive evidence was obtained of the presence of cytase, pectinase, invertase, maltase, and emulsin, but negative results were obtained in the tests for pectase and inulase.

Among the other enzymes found to be very active were the proteases and amidase. The ammonia liberated by the action of the amidase on the amides was probably responsible, at least to some extent, for the effect found ahead of the pioneering mycelial threads in the fruit.

In the diseased fruit the advancing zone which lies between the margin of discoloration and the healthy tissues is mainly due to the accumulation of enzymes and their by-products.

Lipase was present in the ground mycelium and was more active on methyl and ethyl acetates than on lemon-oil emulsion.

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